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(57) Abstract

Oligonucleotides and other macromolecules are provided that have increased nuclease resistance, substituent groups for increasing binding affinity to complementary strand, and subsequences of 2′-deoxy-*erythro*-pentofuranosyl nucleotides that activate RNase H enzyme. Such oligonucleotides and macromolecules are useful for diagnostics and other research purposes, for modulating protein in organisms, and for the diagnosis, detection and treatment of other conditions susceptible to antisense therapeutics.

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GAPPED 2′ MODIFIED OLIGONUCLEOTIDES

FIELD OF THE INVENTION

This invention is directed to the synthesis and use of oligonucleotides and macromolecules to elicit RNase H for strand cleavage in an opposing strand. Included in the invention are oligonucleotides wherein at least some of the nucleotides of the oligonucleotides are functionalized to be nuclease resistant, at least some of the nucleotides of the oligonucleotide include a substituent that potentiates hybridization of the oligonucleotide to a complementary strand, and at least some of the nucleotides of the oligonucleotide include 2′-deoxy-erythro-pentofuranosyl sugar moieties. The oligonucleotides and macromolecules are useful for therapeutics, diagnostics and as research reagents.

BACKGROUND OF THE INVENTION

It is well known that most of the bodily states in mammals including most disease states, are effected by proteins. Such proteins, either acting directly or through their enzymatic functions, contribute in major proportion to many diseases in animals and man. Classical therapeutics has generally focused upon interactions with such proteins in an effort to moderate their disease causing or disease potentiating functions. Recently, however, attempts have been made to moderate the actual production of such proteins by interactions with messenger RNA (mRNA) or other intracellular RNA's that direct protein synthesis. It is generally the object of such therapeutic approaches to interfere with or otherwise modulate gene expression leading to undesired protein

formation.

Antisense methodology is the complementary hybridization of relatively short oligonucleotides to single-stranded RNA or single-stranded DNA such that the normal, essential functions of these intracellular nucleic acids are disrupted. Hybridization is the sequence specific hydrogen bonding via Watson-Crick base pairs of the heterocyclic bases of oligonucleotides to RNA or DNA. Such base pairs are said to be complementary to one another.

Naturally occurring events that provide for the disruption of the nucleic acid function, as discussed by Cohen in *Oligonucleotides: Antisense Inhibitors of Gene Expression*, CRC Press, Inc., Boca Raton, Fl (1989) are thought to be of two types. The first is hybridization arrest. This denotes the terminating event in which an oligonucleotide inhibitor binds to target nucleic acid and thus prevents, by simple steric hindrance, the binding of essential proteins, most often ribosomes, to the nucleic acid. Methyl phosphonate oligonucleotides (*see, e.g.*, Miller, *et al.*, *Anti-Cancer Drug Design* **1987**, *2*, 117) and α-anomer oligonucleotides are the two most extensively studied antisense agents that are thought to disrupt nucleic acid function by hybridization arrest.

In determining the extent of hybridization arrest of an oligonucleotide, the relative ability of an oligonucleotide to bind to complementary nucleic acids may be compared by determining the melting temperature of a particular hybridization complex. The melting temperature (T_m), a characteristic physical property of double helixes, denotes the temperature in degrees centigrade at which 50% helical (hybridized) versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the formation and breakdown (melting) of hybridization. Base stacking which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently a reduction in UV absorption indicates a higher T_m. The higher the T_m, the greater the strength of the binding of the strands. Non-Watson-Crick base pairing, i.e. base mismatch, has a strong

destabilizing effect on the T_m.

The second type of terminating vent for antisense oligonucleotides involves the enzymatic cleavage of the targeted RNA by intracellular RNase H. The mechanism of such RNase H cleavages requires that a 2'-deoxyribofuranosyl oligonucleotide hybridize to a targeted RNA. The resulting DNA-RNA duplex activates the RNase H enzyme; the activated enzyme cleaves the RNA strand. Cleavage of the RNA strand destroys the normal function of the RNA. Phosphorothioate oligonucleotides are one prominent example of antisense agents that operate by this type of terminating event. For a DNA oligonucleotide to be useful for activation of RNase H, the oligonucleotide must be reasonably stable to nucleases in order to survive in a cell for a time sufficient for the RNase H activation.

Several recent publications of Walder, *et al.* further describe the interaction of RNase H and oligonucleotides. Of particular interest are: (1) Dagle, *et al.*, *Nucleic Acids Research* **1990**, 18, 4751; (2) Dagle, *et al.*, *Antisense Research And Development* **1991**, *1*, 11; (3) Eder, *et al.*, *J. Biol. Chem.* **1991**, *266*, 6472; and (4) Dagle, *et al.*, *Nucleic Acids Research* **1991**, *19*, 1805. In these papers, Walder, *et al.* note that DNA oligonucleotides having both unmodified phosphodiester internucleoside linkages and modified, phosphorothioate internucleoside linkages are substrates for cellular RNase H. Since they are substrates, they activate the cleavage of target RNA by the RNase H. However, the authors further note that in *Xenopus* embryos, both phosphodiester linkages and phosphorothioate linkages are also subject to exonuclease degradation. Such nuclease degradation is detrimental since it rapidly depletes the oligonucleotide available for RNase H activation.

As described in references (1), (2), and (4), to stabilize their oligonucleotides against nuclease degradation while still providing for RNase H activation, Walder, *et al.* constructed 2'-deoxy oligonucleotides having a short section of phosphodiester linked nucleotides positioned between sections of phosphoramidate, alkyl phosphonate or phosphotriester

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linkages. While the phosphoamidate-containing oligonucleotides were stabilized against exonucleases, in reference (4) the authors noted that each phosphoramidate linkage resulted in a loss of 1.6°C in the measured T_m value of the phosphoramidate containing oligonucleotides. Such decrease in the T_m value is indicative of an undesirable decrease in the hybridization between the oligonucleotide and its target strand.

Other authors have commented on the effect such a loss of hybridization between an antisense oligonucleotide and its targeted strand can have. Saison-Behmoaras, *et al.*, *EMBO Journal* **1991**, *10*, 1111, observed that even through an oligonucleotide could be a substrate for RNase H, cleavage efficiency by RNase H was low because of weak hybridization to the mRNA. The authors also noted that the inclusion of an acridine substitution at the 3′ end of the oligonucleotide protected the oligonucleotide from exonucleases.

While it has been recognized that cleavage of a target RNA strand using an antisense oligonucleotide and RNase H would be useful, nuclease resistance of the oligonucleotide and fidelity of the hybridization are also of great importance. Heretofore, there have been no suggestion in the art of methods or materials that could both activate RNase H while concurrently maintaining or improving hybridization properties and providing nuclease resistance even though there has been a long felt need for such methods and materials. Accordingly, there remains a long-felt need for such methods and materials.

OBJECTS OF THE INVENTION

It is an object of this invention to provide oligonucleotides that both activate RNase H upon hybridization with a target strand and resist nuclease degradation.

It is a further object to provide oligonucleotides that activate RNase H, inhibit nuclease degradation, and provide improved binding affinity between the oligonucleotide and the target strand.

A still further object is to provide research and diagnostic methods and materials for assaying bodily states in animals, especially diseased states.

Another object is to provide therapeutic and research methods and materials for the treatment of diseases through modulation of the activity of DNA and RNA.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with one embodiment of this invention there are provided oligonucleotides formed from a sequence of nucleotide units. The oligonucleotides incorporate a least one nucleotide unit that is functionalized to increase nuclease resistance of the oligonucleotides. Further, at least some of the nucleotide units of the oligonucleotides are functionalized with a substituent group to increase binding affinity of the oligonucleotides to target RNAs, and at least some of the nucleotide units have 2'-deoxy-erythro-pentofuranosyl sugar moieties.

In preferred oligonucleotides of the invention, nucleotide units that are functionalized for increased binding affinity are functionalized to include a 2'-substituent group. In even more preferred embodiments, the 2'-substituent group is fluoro, C1-C9 alkoxy, C1-C9 aminoalkoxy including aminopropoxy, allyloxy, C_1-C_9-alkyl-imidazole and polyethylene glycol. Preferred alkoxy substituents include methoxy, ethoxy and propoxy. A preferred aminoalkoxy unit is aminopropoxy. A preferred alkyl-imidazole is 1-propyl-3-(imidazoyl).

In certain preferred oligonucleotides of the invention having increased nuclease resistance, each nucleotide unit of

the oligonucleotides is a phosphorothioate or phosphorodithioate nucleotide. In other preferred oligonucleotides, the 3′ terminal nucleotide unit is functionalized with either or both of a 2′ or a 3′ substituent.

The oligonucleotides include a plurality of nucleotide units bearing substituent groups that increase binding affinity of the oligonucleotide to a complementary strand of nucleic acid. In certain preferred embodiments, the nucleotide units that bear such substituents can be divided into a first nucleotide unit sub-sequence and a second nucleotide unit sub-sequence, with 2′-deoxy-erythro-pentofuranosyl structures being positioned within the oligonucleotide between the first nucleotide unit sub-sequence and the second nucleotide unit sub-sequence. It is preferred that all such intervening nucleotide units be 2′-deoxy-erythro-pentofuranosyl units.

In further preferred oligonucleotides of the invention, nucleotide units bearing substituents that increase binding affinity are located at one or both of the 3′ or the 5′ termini of the oligonucleotide. There can be from one to about eight nucleotide units that are substituted with substituent groups. Preferably, at least five sequential nucleotide units are 2′-deoxy-erythro-pentofuranosyl sugar moieties.

The present invention also provides macromolecules formed from a plurality of linked nucleosides selected from α-nucleosides, β-nucleosides including 2′-deoxy-erythro-pentofuranosyl β-nucleosides, 4′-thionucleosides, and carbocyclic-nucleosides. These nucleosides are connected by linkages in a sequence that is hybridizable to a complementary nucleic acid. The linkages are selected from charged phosphorous linkages, neutral phosphorous linkages, and non-phosphorous linkages. The sequence of linked nucleosides is divided into at least two regions. The first nucleoside region includes the following types of nucleosides: α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages; α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages; α-nucleosides linked by non-phosphorous linkages; 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous

linkages; 4′-thionucleosides linked by charged and neutral 2′-5′ phosphorous linkages; 4′-thionucleosides linked by non-phosphorous linkages; carbocyclic-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages; carbocyclic-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages; carbocyclic-nucleosides linked by non-phosphorous linkages; ß-nucleosides linked by charged and neutral 2′-5′ linkages; and ß-nucleosides linked by non-phosphorous linkages. A second nucleoside region consists of 2′-deoxy-erythro-pentofuranosyl ß-nucleosides linked by charged 3′-5′ phosphorous linkages having negative charge at physiological pH. In preferred embodiments, the macromolecules include at least 3 of said 2′-deoxy-erythro-pentofuranosyl ß-nucleosides, more preferably at least 5 of said 2′-deoxy-erythro-pentofuranosyl ß-nucleotides. In further preferred embodiments there exists a third nucleoside region whose nucleosides are selected from those selectable for the first region. In preferred embodiments the second region is positioned between the first and third regions.

Preferred charged phosphorous linkages include phosphodiester, phosphorothioate, phosphorodithioate, phosphoroselenate and phosphorodiselenate linkages; phosphodiester and phosphorothioate linkages are particularly preferred. Preferred neutral phosphorous linkages include alkyl and aryl phosphonates, alkyl and aryl phosphoroamidites, alkyl and aryl phosphotriesters, hydrogen phosphonate and boranophosphate linkages. Preferred non-phosphorous linkages include peptide linkages, hydrazine linkages, hydroxy-amine linkages, carbamate linkages, morpholine linkages, carbonate linkages, amide linkages, oxymethyleneimine linkages, hydrazide linkages, silyl linkages, sulfide linkages, disulfide linkages, sulfone linkages, sulfoxide linkages, sulfonate linkages, sulfonamide linkages, formacetal linkages, thioformacetal linkages, oxime linkages and ethylene glycol linkages.

The invention also provides macromolecules formed from a plurality of linked units, each of which is selected from nucleosides and nucleobases. The nucleosides include α-

nucleosides, ß-nucleosides including 2′-deoxy-erythro-pentofuranosyl ß-nucleosides, 4′-thionucleosides and carbocyclic-nucleosides. The nucleobases include purin-9-yl and pyrimidin-1-yl heterocyclic bases. The nucleosides and nucleobases of the units are linked together by linkages in a sequence wherein the sequence is hybridizable to a complementary nucleic acid and the sequence of linked units is divided into at least two regions. The linkages are selected from charged 3′-5′ phosphorous, neutral 3′-5′ phosphorous, charged 2′-5′ phosphorous, neutral 2′-5′ phosphorous or non-phosphorous linkages. A first of the regions includes nucleobases linked by non-phosphorous linkages and nucleobases that are attached to phosphate linkages via non-sugar tethering groups, and nucleosides selected from α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, α-nucleosides linked by non-phosphorous linkages, 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous linkages, 4′-thionucleosides linked by charged and neutral 2′-5′ phosphorous linkages, 4′-thionucleosides linked by non-phosphorous linkages, carbocyclic-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, carbocyclic-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, carbocyclic-nucleosides linked by non-phosphorous linkages, ß-nucleosides linked by charged and neutral 2′-5′ linkages, and ß-nucleosides linked by non-phosphorous linkages. A second of the regions includes only 2′-deoxy-erythro-pentofuranosyl ß-nucleosides linked by charged 3′-5′ phosphorous linkages wherein the 3′-5′ phosphorous linkages have a negative charge at physiological pH.

In certain preferred embodiments, the first region includes at least two nucleobases joined by a non-phosphate linkage such as a peptide linkage. In preferred embodiments, the macromolecules include a third region that is selected from the same groups as described above for the first region. In preferred embodim nts, the second region is located between the first and third regions.

The invention also provides macromolecules that have a plurality of linked units, each of which is selected from nucleosides and nucleobases. The nucleosides are selected from α-nucleosides, β-nucleosides, 4'-thionucleosides and carbocyclic-nucleosides and the nucleobases are selected from purin-9-yl and pyrimidin-1-yl heterocyclic bases. The nucleosides and nucleobases of said units are linked together by linkages in a sequence wherein the sequence is hybridizable to a complementary nucleic acid. The sequence of linked units is divided into at least two regions. The linkages are selected from charged phosphorous, neutral phosphorous or non-phosphorous linkages. A first of the regions include α-nucleosides linked by charged and neutral 3'-5' phosphorous linkages, α-nucleosides linked by charged and neutral 2'-5' phosphorous linkages, α-nucleosides linked by non-phosphorous linkages, 4'-thionucleosides linked by charged and neutral 3'-5' phosphorous linkages, 4'-thionucleosides linked by charged and neutral 2'-5' phosphorous linkages, 4'-thionucleosides linked by non-phosphorous linkages, carbocyclic-nucleosides linked by charged and neutral phosphorous linkages, carbocyclic-nucleosides linked by non-phosphorous linkages, β-nucleosides linked by charged and neutral 3'-5' linkages, β-nucleosides linked by charged and neutral 2'-5' linkages, and β-nucleosides linked by non-phosphorous linkages. A second of the regions include nucleobases linked by non-phosphorous linkages and nucleobases that are attached to phosphate linkages via a non-sugar tethering moiety.

Preferred nucleobases of the invention include adenine, guanine, cytosine, uracil, thymine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl adenines, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo adenine, 8-amino-adenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8 substituted adenines and 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8 substituted guanines,

other aza and deaza uracils, other aza and deaza thymidines, other aza and deaza cytosine, aza and deaza adenines, aza and deaza guanines or 5-trifluoromethyl uracil and 5-trifluorocytosine.

The invention also provides methods of treating an organism having a disease characterized by the undesired production of an protein. These methods include contacting the organism with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a complementary strand of nucleic acid where at least one of the nucleotides is functionalized to increase nuclease resistance of the oligonucleotide to nucleases, where a substituent group located thereon to increase binding affinity of the oligonucleotide to the complementary strand of nucleic acid and where a plurality of the nucleotides have 2′-deoxy-erythroregions;-pentofuranosyl sugar moieties.

Further in accordance with this invention there are provided compositions including a pharmaceutically effective amount of an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a complementary strand of nucleic acid and where at least one of the nucleotides is functionalized to increase nuclease resistance of the oligonucleotide to nucleases and where a plurality of the nucleotides have a substituent group located thereon to increase binding affinity of the oligonucleotide to the complementary strand of nucleic acid and where a plurality of the nucleotides have 2′-deoxy-erythro-pentofuranosyl sugar moieties. The composition further include a pharmaceutically acceptable diluent or carrier.

Further in accordance with this invention there are provided methods for *in vitro* modification of a sequence specific nucleic acid including contacting a test solution containing an RNase H enzyme and said nucleic acid with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a complementary strand of nucleic acid and where at least one of the nucleotides is functionalized to increase nuclease resistance of the

oligonucleotide to nucl ases and where a plurality of the nucleotides have a substituent group located thereon to increase binding affinity of the oligonucleotide to the complementary strand of nucleic acid and where a plurality of the nucleotides have 2'-deoxy-erythro-pentofuranosyl sugar moieties.

There are also provided methods of concurrently enhancing hybridization and RNase H enzyme activation in an organism that includes contacting the organism with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a complementary strand of nucleic acid and where at least one of the nucleotides is functionalized to increase nuclease resistance of the oligonucleotide to nucleases and where a plurality of the nucleotides have a substituent group located thereon to increase binding affinity of the oligonucleotide to the complementary strand of nucleic acid and where a plurality of the nucleotides have 2'-deoxy-erythro-pentofuranosyl sugar moieties.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be better understood when taken in conjunction with the drawings wherein:

Figure 1 is a graph showing dose response activity of oligonucleotides of the invention and a reference compound; and

Figure 2 is a bar chart showing dose response activity of oligonucleotides of the invention and reference compounds.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the objects of this invention, novel oligonucleotides and macromolecules that, at once, have increased nuclease resistance, increased binding affinity to complementary strands and that are substrates for RNase H are provided. The oligonucleotides and macromolecules of the invention are assembled from a plurality of nucleotide, nucleoside or nucleobase sub-units. Each oligonucleotide or macromol cule of the invention includes at least one

nucleotide, nucleoside or nucleobase unit that is functionalized to increase the nuclease resistances of the oligonucleotide. Further, in certain embodiments of the invention at least some of the nucleotide or nucleoside units bear a substituent group that increases the binding affinity of the oligonucleotide or macromolecule to a complementary strand of nucleic acid. Additionally at least some of the nucleotide units comprise a 2′-deoxy-erythro-pentofuranosyl group as their sugar moiety.

In conjunction with the above guidelines, each nucleotide unit of an oligonucleotides of the invention, alternatively referred to as a subunit, can be a "natural" or a "synthetic" moiety. Thus, in the context of this invention, the term "oligonucleotide" in a first instance refers to a polynucleotide formed from a plurality of joined nucleotide units. The nucleotides units are joined together via native internucleoside, phosphodiester linkages. The nucleotide units are formed from naturally-occurring bases and pentofuranosyl sugars groups. The term "oligonucleotide" thus effectively includes naturally occurring species or synthetic species formed from naturally occurring nucleotide units.

Oligonucleotides of the invention also can include modified subunits. The modifications can occur on the base portion of a nucleotide, on the sugar portion of a nucleotide or on the linkage joining one nucleotide to the next. In addition, nucleoside units can be joined via connecting groups that substitute for the inter-nucleoside phosphate linkages. Macromolecules of the type have been identified as oligonucleosides. In such oligonucleosides the linkages include an $-O-CH_2-CH_2-O-$ linkage (*i.e.*, an ethylene glycol linkage) as well as other novel linkages disclosed in the following United States patent applications: Serial Number 566,836, filed August 13, 1990, entitled Novel Nucleoside Analogs; Serial Number 703,619, filed May 21, 1991, entitled Backbone Modified Oligonucleotide Analogs; and Serial Number 903,160, filed June 24, 1992, entitled Heteroatomic Oligonucleotide Linkage. Other modifications can be made to

the sugar, to the base, or to the phosphate group of the nucleotide. Representative modifications are disclosed in the following United States patent applications: Serial Number 463,358, filed January 11, 1990, entitled Compositions And Methods For Detecting And Modulating RNA Activity; Serial Number 566,977, filed August 13, 1990, entitled Sugar Modified Oligonucleotides That Detect And Modulate Gene Expression; Serial Number 558,663, filed July 27, 1990, entitled Novel Polyamine Conjugated Oligonucleotides; Serial Number 558,806, filed July 27, 1991, entitled Nuclease Resistant Pyrimidine Modified Oligonucleotides That Detect And Modulate Gene Expression; and Serial Number PCT/US91/00243, filed January 11, 1991, entitled Compositions and Methods For Detecting And Modulating RNA Activity, all assigned to the assignee of this invention. The disclosures of each of the above noted patent applications are herein incorporated by reference.

Thus, the terms oligonucleotide is intended to include naturally occurring structures as well as non-naturally occurring or "modified" structures -- including modified sugar moieties, modified base moieties or modified sugar linking moieties -- that function similarly to natural bases, natural sugars and natural phosphodiester linkages. Thus, oligonucleotides can have altered base moieties, altered sugar moieties or altered inter-sugar linkages. Exemplary among these are phosphorothioate, phosphorodithioate, methyl phosphonate, phosphotriester, phosphoramidate, phosphoroselenate and phosphorodiselenate inter-nucleoside linkages used in place of phosphodiester inter-nucleoside linkages; deaza or aza purines and pyrimidines used in place of natural purine and pyrimidine bases; pyrimidine bases having substituent groups at the 5 or 6 position; purine bases having altered or replacement substituent groups at the 2, 6 or 8 positions; or sugars having substituent groups at their 2′ position, substitutions for one or more of the hydrogen atoms of the sugar, or carbocyclic or acyclic sugar analogs. They may also comprise other modifications consistent with the spirit of this invention. Such oligonucleotides are best described as being functionally

interchangeable with natural oligonucleotides (or synthesized oligonucleotides along natural lines), but which have one or more differences from natural structure. All such oligonucleotides are comprehended by this invention so long as they function effectively to mimic the structure of a desired RNA or DNA strand.

In one preferred embodiment of this invention, nuclease resistance is achieved by utilizing phosphorothioate internucleoside linkages. Contrary to the reports of Walder, *et al.* note above, I have found that in systems such as fetal calf serum containing a variety of 3′-exonucleases, modification of the internucleoside linkage from a phosphodiester linkage to a phosphorothioate linkage provides nuclease resistance.

Brill, *et al.*, *J. Am. Chem. Soc.* **1991**, *113*, 3972, recently reported that phosphorodithioate oligonucleotides also exhibit nuclease resistance. These authors also reported that phosphorodithioate oligonucleotide bind with complementary deoxyoligonucleotides, stimulate RNase H and stimulate the binding of *lac* repressor and *cro* repressor. In view of these properties, phosphorodithioates linkages also may be useful to increase nuclease resistance of oligonucleotides of the invention.

Nuclease resistance further can be achieved by locating a group at the 3′ terminus of the oligonucleotide utilizing the methods of Saison-Behmoraras, *et al.*, *supra*, wherein a dodecanol group is attached to the 3′ terminus of the oligonucleotide. Other suitable groups for providing increased nuclease resistance may include steroid molecules and other lipids, reporter molecules, conjugates and non-aromatic lipophilic molecules including alicyclic hydrocarbons, saturated and unsaturated fatty acids, waxes, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes. Particularly useful as steroid molecules for this purpose are the bile acids including cholic acid, deoxycholic acid and dehydrocholic acid. Other steroids include cortisone, digoxigenin, testosterone and cholesterol

and even cationic steroids such as cortisone having a trimethylaminomethyl hydrazide group attached via a double bond at the 3 position of the cortisone ring. Particularly useful reporter molecules are biotin and fluorescein dyes. Such groups can be attached to the 2′ hydroxyl group or 3′ hydroxyl group of the 3′ terminal nucleotide either directly or utilizing an appropriate connector in the manner described in United States Patent Application Serial Number 782,374, filed October 24, 1991 entitled Derivatized Oligonucleotides Having Improved Uptake and Other Properties, assigned to the assignee as this application, the entire contents of which are herein incorporated by reference.

Attachment of functional groups at the 5′ terminus of compounds of the invention also may contribute to nuclease resistance. Such groups include acridine groups (which also serves as an intercalator) or other groups that exhibit either beneficial pharmacokinetic or pharmacodynamic properties. Groups that exhibit pharmacodynamic properties, in the context of this invention, include groups that improve oligonucleotide uptake, enhance oligonucleotide resistance to degradation, and/or strengthened sequence-specific hybridization with RNA. Groups that exhibit pharmacokinetic properties, in the context of this invention, include groups that improve oligonucleotide uptake, distribution, metabolism or excretion.

Further nuclease resistance is expect to be conferred utilizing linkages such as the above identified $-O-CH_2-CH_2-O-$ linkage and similar linkages of the above identified United State Patent Applications Serial Number 566,836, Serial Number 703,619, and Serial Number 903,160, since these types of linkages do not utilize natural phosphate ester-containing backbones that are the natural substrates for nucleases. When nuclease resistance is conferred upon an oligonucleotide of the invention by the use of a phosphorothioate or other nuclease resistant internucleotide linkages, such linkages will reside in each internucleotide sites. In other embodiments, less than all of the internucleotide linkages will be modified to phosphorothioate or other nuclease resistant linkages.

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I have found that binding affinity of oligonucleotides of the invention can be increased by locating substituent groups on nucleotide subunits of the oligonucleotides of the invention. Preferred substituent groups are 2′ substituent groups, *i.e.*, substituent groups located at the 2′ position of the sugar moiety of the nucleotide subunits of the oligonucleotides of the invention. Presently preferred substituent groups include but are not limited to 2′-fluoro, 2′-alkoxy, 2′-aminoalkoxy, 2′-allyloxy, 2′-imidazole-alkoxy and 2′-poly(ethylene oxide). Alkoxy and aminoalkoxy groups generally include lower alkyl groups, particularly C1-C9 alkyl. Poly(ethylene glycols) are of the structure $(O\text{-}CH_2\text{-}CH_2)_n$-O-alkyl. Particularly preferred substituent groups are 2′-fluoro, 2′-methoxy, 2′-ethoxy, 2′-propoxy, 2′-aminopropoxy, 2′-imidazolepropoxy, 2′-imidazolebutoxy, and 2′-allyloxy groups.

Binding affinity also can be increased by the use of certain modified bases in the nucleotide units that make up the oligonucleotides of the invention. Such modified bases may include 6-azapyrimidines and N-2, N-6 and O-6 substituted purines including 2-aminopropyladenine. Other modified pyrimidine and purine base are expected to increase the binding affinity of oligonucleotides to a complementary strand of nucleic acid.

The use of 2′-substituent groups increases the binding affinity of the substituted oligonucleotides of the invention. In a published study, Kawasaki and Cook, *et al.*, *Synthesis and Biophysical Studies of 2′-dRIBO-F Modified Oligonucleotides*, Conference On Nucleic Acid Therapeutics, Clearwater, FL, January 13, 1991, the inventor has reported a binding affinity increase of 1.6°C per substituted nucleotide unit of the oligonucleotide. This is compared to an unsubstituted oligonucleotide for a 15 mer phosphodiester oligonucleotide having 2′-deoxy-2′-fluoro groups as a substituent group on five of the nucleotides of the oligonucleotide. When 11 of the nucleotides of the oligonucleotide bore such 2′-deoxy-2′-fluoro substituent groups, the binding affinity increased to 1.8°C per substituted nucleotide unit.

In that same study, the 15 mer phosphodiester oligonucleotide was derivatized to the corresponding phosphorothioate analog. When the 15 mer phosphodiester oligonucleotide was compared to its phosphorothioate analog, the phosphorothioate analog had a binding affinity of only about 66% of that of the 15 mer phosphodiester oligonucleotide. Stated otherwise, binding affinity was lost in derivatizing the oligonucleotide to its phosphorothioate analog. However, when 2'-deoxy-2'-fluoro substituents were located at 11 of the nucleotides of the 15 mer phosphorothioate oligonucleotide, the binding affinity of the 2'-substituent groups more than overcame the decrease noted by derivatizing the 15 mer oligonucleotide to its phosphorothioate analog. In this compound, *i.e.*, a 15 mer phosphorothioate oligonucleotide having 11 nucleotide substituted with 2'-fluoro groups, the binding affinity was increased to 2.5°C per substituent group. In this study no attempt was made to include an appropriate consecutive sequence of nucleotides have 2'-deoxy-erythro-pentofuranosyl sugars that would elicit RNase H enzyme cleavage of a RNA target complementary to the oligonucleotide of the study.

In order to elicit RNase H enzyme cleavage of a target RNA, an oligonucleotide of the invention must include a segment or sub-sequence therein that is a DNA type segment. Stated otherwise, at least some of the nucleotide subunits of the oligonucleotides of the invention must have 2'-deoxy-erythro-pentofuranosyl sugar moieties. I have found that a sub-sequence having more than three consecutive, linked 2'-deoxy-erythro-pentofuranosyl-containing nucleotide sub-units likely is necessary in order to elicit RNase H activity upon hybridization of an oligonucleotide of the invention with a target RNA. It is presently preferred to have a sub-sequence of 5 or more consecutive 2'-deoxy-erythro-pentofuranosyl containing nucleotide subunits in an oligonucleotide of the invention. Use of at least 7 consecutive 2'-deoxy-erythro-pentofuranosyl-containing nucleotide subunits is particularly preferred.

The mechanism of action of RNase H is recognition of a DNA-RNA duplex followed by cleavage of the RNA stand of this

duplex. As noted in the Background section above, others in the art have used modified DNA strands to impart nuclease stability to the DNA strand. To do this they have used modified phosphate linkages impart increased nuclease stability but detract from hybridization properties. While I do not wish to be bound by theory, I have identified certain nucleosides or nucleoside analogs that will impart nuclease stability to an oligonucleotide, oligonucleoside or other macromolecule and in certain instances also impart increase binding to a complementary strand. These include α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, α-nucleosides linked by non-phosphorous linkages, 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous linkages, 4′-thionucleosides linked by charged and neutral 2′-5′ phosphorous linkages, 4′-thionucleosides linked by non-phosphorous linkages, carbocyclic-nucleosides linked by charged and neutral phosphorous linkages, carbocyclic-nucleosides linked by non-phosphorous linkages, ß-nucleosides linked by charged and neutral 3′-5′ linkages, ß-nucleosides linked by charged and neutral 2′-5′ linkages, and ß-nucleosides linked by non-phosphorous linkages. They further include nucleobases that are attached to phosphate linkages via non-sugar tethering groups or are attached to non-phosphate linkages.

Again, while not wishing to be bound by any particular theory, I have found certain criteria that must be met for RNase H to recognize and elicit cleavage of a RNA strand. The first of these is that the RNA stand at the cleavage site must have its nucleosides connected via a phosphate linkage that bears a negative charge. Additionally, the sugar of the nucleosides at the cleavage site must be a ß-pentofuranosyl sugar and also must be in a 2′ endo conformation. The only nucleosides (nucleotides) that fit this criteria are phosphodiester, phosphorothioate, phosphorodithioate, phosphoroselenate and phosphorodiselenate nucleotides of 2′-deoxy-erythro-pentofuranosyl ß-nucleosides.

In view of the above criteria, ven certain

nucleosides that have been shown to reside in a 2′ endo conformation (*e.g.*, cyclopentyl nucleosides) will not elicit RNase H activity since they do not incorporate a pentofuranosyl sugar. Modeling has shown that oligonucleotide 4′-thionucleosides also will not elicit RNase H activity, even though such nucleosides reside in an envelope conformation, since they do not reside in a 2′ endo conformation. Additionally, since α-nucleosides are of the opposite configuration from β-pentofuranosyl sugars they also will not elicit RNase H activity.

Nucleobases that are attached to phosphate linkages via non-sugar tethering groups or via non-phosphate linkages also do not meet the criteria of having a β-pentofuranosyl sugar in a 2′ endo conformation. Thus, they likely will not elicit RNase H activity.

As used herein, α and β nucleosides include ribofuranosyl, deoxyribofuranosyl (2′-deoxy-erythro-pentofuranosyl) and arabinofuranosyl nucleosides. 4′-Thionucleosides are nucleosides wherein the 4′ ring oxygen atom of the pentofuranosyl ring is substituted by a sulfur atom. Carbocyclic nucleosides are nucleosides wherein the ring oxygen is substituted by a carbon atom. Carbocyclic nucleosides include cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl rings (C_3-C_6-carbocyclic) having an appropriate nucleobase attached thereto. The above α and β nucleosides, 4′-thionucleosides and carbocyclic nucleosides can include additional functional groups on their heterocyclic base moiety and additional functional groups on those carbon atoms of sugar or carbocyclic moiety that are not utilized in linking the nucleoside in a macromolecule of the invention. For example, substituent groups can be placed on the 1, 2, 3, 6, 7 or 8 position of purine heterocycles, the 2, 3, 4, 5 or 6 position of pyrimidine heterocycles. Deaza and aza analogs of the purine and pyrimidine heterocycles can be selected or 2′ substituted sugar derivatives can be selected. All of these types of substitutions are known in the nucleoside art.

α-Nucleosides have been incorporated into oligo-

nucleotides; as reported by Gagnor, *et. al.*, *Nucleic Acids Research* **1987**, *15*, 10419, they do not support RNase H degradation. Carbocyclic modified oligonucleotides have been synthesized by a number of investigators, including Perbost, *et al.*, *Biochemical and Biophysical Research Communications* **1989**, *165*, 742; Sagi, *et al.*, *Nucleic Acids Research* **1990**, *18*, 2133; and Szemzo, *et. al.*, *Tetrahedron Letters* **1990**, *31*, 1463. 4′-Thionucleosides have been known for at least 25 years. An improved synthesis via 4′-thioribofuranose recently was reported by Secrist, *et. al.*, *Tenth International Roundtable: Nucleosides, Nucleotides and Their Biological Evaluation*, September 16-20, 1992, Abstracts of Papers, Abstract 21 and in published patent application PCT/US91/02732.

For incorporation into oligonucleotides or oligonucleotide suggorates, α and ß nucleosides, 4′-thionucleosides and carbocyclic nucleosides will be blocked in the 5′ position (or the equivalent to the 5′ position for the carbocyclic nucleosides) with a dimethoxytrityl group, followed by phosphitylation in the 3′ position as per the tritylation and phosphitylation procedures reported in *Oligonucleotides and Analogs, A Practical Approach*, Eckstein, F., Ed.; The Practical Approach Series, IRL Press, New York, 1991. Incorporation into oligonucleotides will be accomplished utilizing a DNA synthesizer such as an ABI 380 B model synthesizer using appropriate chemistry for the formation of phosphodiester, phosphorothioate, phosphorodithioate or methylphosphonates as per the synthetic protocols illustrated in Eckstein op. cit.

Boranophosphate linked oligonucleotides are prepared as per the methods described in published patent application PCT/US/06949. Phosphoroselenates and phosphorodiselenates linked oligonucleotides are prepared in a manner analogous to their thio counterparts using the reagent 3*H*-1,2-benzothiaseleno-3-ol for introducing the seleno moiety. This reagent is also useful for preparing selenothio-phosphates from corresponding H-phosphonothiate diester as reported by Stawinski, *et al.* *Tenth International Roundtable: Nucleosides, Nucleotides*

and Their Biological Evaluation, September 16-20, 1992, Abstracts of Papers, Abstract 80. Hydrogen phosphonate-linked oligonucleotides -- as well as alkyl and aryl phosphonate, alkyl and aryl phosphotriesters and alkyl and aryl phosphoramidates linked oligonucleotides -- are prepared in the manner of published patent application PCT/US88/03842. This patent application also discusses the preparation of phosphorothioates and phosphoroselenates linked oligonucleotides

Non-phosphate backbones include carbonate, carbamate, silyl, sulfide, sulfone, sulfoxide, sulfonate, sulfonamide, formacetal, thioformacetal, oxime, hydroxylamine, hydrazine, hydrazide, disulfide, amide, urea and peptide linkages. Oligonucleoside having their nucleosides connected by carbonate linkages are prepared as described by, for example, Mertes, *et al.*, *J. Med. Chem.* **1969**, *12*, 154 and later by others. Oligonucleoside having their nucleosides connected by carbamate linkages are prepared as was first described by Gait, *et. al.*, *J. Chem. Soc. Perkin 1* **1974**, 1684 and later by others. Oligonucleoside having their nucleosides connect by silyl linkages are prepared as described Ogilvie, *et al.*, *Tetrahedron Letters* **1985**, *26*, 4159 and *Nucleic Acids Res*. **1988**, *16*, 4583. Oligonucleoside having their nucleosides connected by sulfide linkages and the associated sulfoxide and sulfone linkages are prepared as described by Schneider, *et al.*, *Tetrahedron Letters* **1990**, *31*, 335 and in other publications such as published patent application PCT/US89/02323.

Oligonucleoside having their nucleosides connected by sulfonate linkages are prepared as described by Musicki, *et al.*, *Org. Chem.* **1991**, *55*, 4231 and *Tetrahedron Letters* **1991**, *32*, 2385. Oligonucleoside having their nucleosides connected by sulfonamide linkages are prepared as described by Kirshenbaum, *et. al.*, *The 5th San Diego Conference: Nucleic Acids: New Frontiers*, Poster abstract 28, November 14-16, **1990**. Oligonucleoside having their nucleosides connected by formacetals are prepared as described by Matteucci, *Tetrahedron Letters* **1990**, *31*, 2385 and Veeneman, *et. al.*, *Recueil des Trav.*

Chim. **1990**, *109*, 449 as well as by the procedures of published patent application PCT/US90/06110. Oligonucleoside having their nucleosides connected by thioformacetals are prepared as described by Matteucci, *et. al.*, *J. Am. Chem. Soc.* **1991**, *113*, 7767; Matteucci, *Nucleosides & Nucleotides* **1991**, *10*, 231, and the above noted patent application PCT/US90/06110.

Oligonucleoside having their nucleosides connected by oxime, hydroxylamine, hydrazine and amide linkages will be prepared as per the disclosures of United States Patent Application Serial Number 703,619 filed May 21, 1991 and related PCT patent applications PCT/US92/04292 and PCT/US92/04305 as well as corresponding published procedures by myself and co-authors in Vasseur, *et. al.*, *J. Am. Chem. Soc.* **1992**, *114*, 4006 and Debart, *et. al.*, *Tetrahedron Letters* **1992**, *33*, 2645. Oligonucleoside having their nucleosides connect by morpholine linkages will be prepared as described in United States Patent Number 5,034,506.

Further non-phosphate linkage suitable for use in this invention include linkages have two adjacent heteroatoms in combination with one or two methylene moieties. Oligonucleosides having their nucleosides connect by such linkages will be prepared as per the disclosures of United States patent application serial number 903,160, filed June 24, 1992, the entire disclosure of which is herein incorporated by reference.

Structural units having nucleobases attached via non-phosphate linkages wherein the non-phosphate linkages are peptide linkages will be prepared as per the procedures of patent application PCT/EP/01219. For use in preparing such structural units, suitable nucleobase include adenine, guanine, cytosine, uracil, thymine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo, amino, thiol, thiolalkyl, hydroxyl and other 8 substituted adenines and guanines, 5-trifluoromethyl and other 5 substituted uracils and cytosines, 7-methylguanine and other

nucleobase such as those disclosed in United States Patent Number 3,687,808.

Peptide linkages include 5, 6 and 7 atom long backbones connected by amide links. Other, similar non-phosphate backbones having ester, amide and hydrazide links are prepared as per published patent applications PCT/US86/00544 and PCT/US86/00545.

Other α and ß nucleosides, 4'-thionucleoside and carbocyclic nucleosides having the heterocyclic bases as disclosed for the nucleobases above can be prepared and incorporated in to the respective α and ß nucleosides, 4'-thionucleoside and carbocyclic nucleosides.

Non-sugar tethering groups include 3,4-dihydroxybutyl (*see*, Augustyns, *et. al.*, *Nucleic Acids Research* **1991**, *19*, 2587) and dihydroxyproproxymethyl (*see*, Schneider, *et al.*, *J. Am. Chem. Soc.* **1990**, *112*, 453) and other linear chains such as C_1-C_{10} alkyl, alkenyl and alkynyl. While the 3,4-dihydroxybutyl and dihydroxyproproxymethyl non-sugar tethering groups are the acyclic fragments of a ß-pentofuranosyl sugar, they will not serve to elicit RNase H activation. Preferred for a non-sugar tethering groups is the 3,4-dihydroxybutyl groups since the dihydroxyproproxymethyl when used in an oligonucleotide analog upon hybridization has shown a suppression of the melting temperature between it and a complementary nucleic strand.

Normal 3'-5' phosphodiester linkages of natural nucleic acids have 3 hetero atoms (-O-P-O-) between the respective sugars of the adjacent nucleosides. If the 5' methylene group (the 5' CH_2 group of the 3' nucleoside of the adjacent nucleosides) is also included, these phosphodiester linked nucleic acids can be viewed as being connected via linkages that are 4 atoms long.

Two strands of ß-oligonucleotides will hybridize with each other with an anti-parallel polarity while a strand of α-oligonucleotides will hybridize with strand of ß-oligonucleotides with a parallel polarity. In certain embodiments, oligonucleotid s of the invention will have a region formed of α-nucleotides and a further region formed of ß-nucleotides.

These two regions are connected via an inter-region linkage. For such an oligonucleotide to bind to a corresponding complementary β strand of a nucleic acid and maintain the parallel polarity of the α region simultaneously with the anti-parallel polarity of the β region, either a 3′-3′ connection or a 5′-5′ connection must be made between the α and β regions of the oligonucleotide of the invention. The 3′-3′ connection (having no 5′ methylene moieties) yields a 3 atom long linkage, while the 5′-5′ connection (having two 5′ methylene moieties) yields a 5 atom long linkage.

For embodiments of the invention wherein a 4 atom long linkage between adjacent α and β regions is desired, use of a symmetrical linking nucleoside or nucleoside surrogate will yield a 4 atom long linkage between each adjacent nucleoside pair. An example of such a symmetrical linking nucleoside surrogate is a 3,3-*bis*-hydroxylmethyl cyclobutyl nucleoside as disclosed in my United States Patent Application Serial Number 808,201, filed December 13, 1991, entitled Cyclobutyl Oligonucleotide Surrogates, the entire disclosure of which is herein incorporated by reference.

Other suitable linkages to achieve 4 atom spacing will include alicyclic compounds of the class 1-hydroxyl-2-hydroxylmethyl-alk-ω-yl type moieties wherein a nucleobase is connected to the ω (omega or last) position. Examples of this type of linkage are 9-(1-hydroxyl-2-methylhydroxyl-pent-5-yl)adenine, 9-(1-hydroxyl-2-methylhydroxyl-pent-5-yl)guanine, 1-(1-hydroxyl-2-methylhydroxyl-pent-5-yl)uridine, 1-(1-hydroxyl-2-methylhydroxyl-pent-5-yl)cytosine and the corresponding 3, 4 and 7 atom analogs, wherein a propyl, butyl or hexyl alkyl group is utilized in place of the pentyl group. A further example includes a nucleoside having a pentofuranosyl sugar that is substituted with a 4′-hydroxylmethy group. In this instance the linkages to the 5′ nucleoside is an normal linkage via the normal 5′ hydroxyl moiety, whereas the linkage to the 3′ nucleoside is not through the normal 3′-hydroxyl group but is through the 4′-hydroxylmethy moiety. As with the cyclobutyl nucleoside, with both the alicyclic moieties or the 4′-

substituted nucleoside moieties, a 4 atom long linkage is achieved between adjacent regions of the oligonucleotide of the invention.

In a manner similar to that described above, in those embodiments of this invention that have adjacent regions of a macromolecule formed from different types of moieties, an interconnection of a desired length can be formed between each of the two adjacent regions of the macromolecule. The symmetrical interconnection is achieved by selecting a linking moiety that can form a covalent bond to both of the different types of moieties forming the adjacent regions. The linking moiety is selected such that the resulting chain of atoms between the linking moiety and the different types of moieties is of the same length.

The oligonucleotides and macromolecules of the invention preferably comprise from about 10 to about 30 nucleotide or nucleobase subunits. It is more preferred that such oligonucleotides and macromolecules comprise from about 15 to about 25 subunits. As will be appreciated, a subunit is a base and sugar combination suitably bound to adjacent subunits through phosphorothioate or other linkages or a nucleobase and appropriate tether suitable bound to adjacent subunits through phosphorous or non-phosphorous linkages. Such terms are used interchangeably with the term "unit." In order to elicit a RNase H response, as specified above, within this total overall sequence length of the oligonucleotide or macromolecule will be a sub-sequence of greater than 3 but preferably five or more consecutive 2'-deoxy-erythro-pentofuranosyl containing nucleotide subunits.

It is presently preferred to incorporated the 2'-deoxy-erythro-pentofuranosyl-containing nucleotide sub-sequence within the oligonucleotide or macromolecule main sequence such that within the oligonucleotide or macromolecule other nucleotide subunits of the oligonucleotide or macromolecule are located on either side of the 2'-deoxy-erythro-pentofuranosyl nucleotide sub-sequ nce.

In certain embodiments of the invention, if the

remainder of the nucleotide subunits each include a 2'-substituent group for increased binding affinity, then the 2'-deoxy-erythro-pentofuranosyl nucleotide sub-sequence will be located between a first sub-sequence of nucleotide subunits having 2'-substituent groups and a second sub-sequence of nucleotide subunits having 2'-substituent groups. Other constructions are also possible, including locating the 2'-deoxy-erythro-pentofuranosyl nucleotide sub-sequence at either the 3' or the 5' terminus of the oligonucleotide of the invention.

Compounds of the invention can be utilized in diagnostics, therapeutics and as research reagents and kits. They can be utilized in pharmaceutical compositions by including an effective amount of oligonucleotide of the invention admixed with a suitable pharmaceutically acceptable diluent or carrier. They further can be used for treating organisms having a disease characterized by the undesired production of a protein. The organism can be contacted with an oligonucleotide of the invention having a sequence that is capable of specifically hybridizing with a strand of nucleic acid that codes for the undesirable protein.

Such therapeutic treatment can be practiced in a variety of organisms ranging from unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes DNA-RNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular control is susceptible to such therapeutic and/or prophylactic treatment. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, all plant and all higher animal forms, including warm-blooded animals, can be treated by this therapy. Further, since each of the cells of multicellular eukaryotes also includes both DNA-RNA transcription and RNA-protein translation as an integral part of their cellular activity, such therapeutics and/or diagnostics can also be practiced on such cellular populations. Furthermore, many of the organelles, *e.g.*, mitochondria and chloroplasts, of eukaryotic cells also include transcription

and translation mechanisms. As such, single cells, cellular populations or organelles also can be included within the definition of organisms that are capable of being treated with the therapeutic or diagnostic oligonucleotides of the invention. As used herein, therapeutics is meant to include both the eradication of a disease state, killing of an organism, *e.g.*, bacterial, protozoan or other infection, or control of erratic or harmful cellular growth or expression.

For purpose of illustration, the compounds of the invention have been used in a ras-luciferase fusion system using ras-luciferase transactivation. As described in United States Patent Application Serial Number 07/715,196, filed June 14, 1991, entitled Antisense Inhibition of RAS Oncogene and assigned commonly with this application, the entire contents of which are herein incorporated by reference, the ras oncogenes are members of a gene family that encode related proteins that are localized to the inner face of the plasma membrane. Ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. This single amino acid change is thought to abolish normal control of ras protein function, thereby

converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The following examples and procedures illustrate the present invention and are not intended to limit the same.

EXAMPLE 1

Oligonucleotide synthesis:

Unsubstituted and substituted oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the step wise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides and phosphorothioates were judged from polyacrylamide gel electrophoresis to be greater than 80% full-length material.

EXAMPLE 2

Oligonucleotide Having α Oligonucleotide Regions Flanking Central ß Oligonucleotide Region

A. α-ß Mixed oligonucleotide having non-symmetrical 3′-3′ and 5′-5′ linkages

For the preparation of a 15 mer, a first region 4 nucleotides long of an α oligonucleotide is prepared as per the method of Gagnor, *et. al.*, *Nucleic Acids Research* **1987**, *15*, 10419 or on a DNA synthesizer utilizing the general protocols of Example 1. Preparation is from the 5′ direction towards the 3′ direction. The terminal 3′ hydroxyl groups is deprotected.

A normal ß region of a DNA oligonucleotide 7 nucleotides long is added in a 3′ to 5′ direction terminating in a free 5′ hydroxyl group. A further 4 nucleotide long region of α nucleotides is then added in a 5′ to 3′ direction. The resulting 15 mer mixed α-ß-α oligonucleotide includes a 3 atom 3′-3′ linkage between the first α region and the ß region and a 5 atom 5′-5′ linkage between the second α region and the ß region.

B. α-ß Mixed oligonucleotide having non-symmetrical 3′-3′ and 5′-5′ linkages

The procedure of Example 2-A is repeated except the intermediate ß region is added as a phosphorothioate region by substitution a thiation step for the normal oxidization step. Thiation is conducted via use of the Beaucage Reagent, *i.e.*, the 1,2-benzodithiole-3-one 1,1-dioxide of Example 1.

C. α-ß Mixed oligonucleotide having symmetrical 4 atom linkages

For the preparation of a 17 mer, a first region 4 nucleotides long is of an α-oligonucleotide is prepared on the DNA synthesizer as per the method of Gagnor, *et. al.*, *Nucleic Acids Research* **1987**, *15*, 10419. Preparation is from the 5′ direction towards the 3′ direction. The terminal 3′ hydroxyl groups is deprotected. A single nucleoside surrogate unit, 1α-thymidyl-3ß-hydroxymethyl-3α-methoxytrityloxymethyl-cyclobutane amidite (prepared as per United States Patent Application Serial Number 808,201, identified above) is condensed on the terminal 3′ hydroxyl group of the α-oligonucleotide region in the normal manner as per Example 1. The trityl hydroxyl group blocking group of the cyclobutyl thymidine nucleoside surrogate is deblocked. A 7 nucleotide region of phosphorothioate 2′-deoxy ß-nucleotide sequence is added on the synthesizer. Upon completion of the DNA region of the macromolecule a 1α-thymidyl-2ß-hydroxy-3α-methoxytrityloxycyclobutane unit activated as a normal phosphoramidite on the 2 hydroxy will be condensed on the growing macromolecule in the same manner as is the 1α-thymidyl-3ß-hydroxymethyl-3α-methoxytrityloxymethyl-cyclobutane moiety above. Following deblocking of the trityl

blocking group of the nucleoside surrogate unit, a further 4 nucleotide stretch of α-oligonucleotides is added to complete the macromolecule. Deblocking, removal from the support and purification of the resulting macromolecule is conducted in the normal manner.

EXAMPLE 3

Oligonucleotide Having 2′-Substituted Oligonucleotides Regions Flanking Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A 15 mer RNA target of the sequence 5′GCG TTT TTT TTT TGC G 3′ was prepared in the normal manner on the DNA sequencer using RNA protocols. A series of phosphorothioate complementary oligonucleotides having 2′-O-substituted nucleotides in regions that flank 2′-deoxy region are prepared utilizing 2′-O-substituted nucleotide precursor prepared as per known literature preparations, *i.e.*, 2′-O-methyl, or as per the procedures of PCT application PCT/US91/05720 or United States Patent Applications 566,977 or 918,362. The 2′-O-substituted nucleotides are added as their 5′-O-dimethoxytrityl-3′-phosphoramidites in the normal manner on the DNA synthesizer. The complementary oligonucleotides have the sequence of 5′ CGC AAA AAA AAA AAA ACG C 3′. The 2′-O-substituent was located in CGC and CG regions of these oligonucleotides. The following 2′-O-substituents are used: 2′-fluoro; 2′-O-methyl; 2′-O-propyl; 2′-O-allyl; 2′-O-aminopropoxy; 2′-O-(methoxyethoxyethyl), 2′-O-imidazolebutoxy and 2′-O-imidazolepropoxy. Additionally the same sequence is prepared in both as a phosphodiester and a phosphorothioate. Following synthesis the test compounds and the target compound are subjected to a melt analysis to measure their Tm′s and nuclease resistance as per the protocols in the above referenced PCT application PCT/US91/05720. The test sequences were found not be substrates for RNase H whereas as the corresponding target sequence is. These test sequences will be nuclease stable and will have increase binding affinity to the target compared to the phosphodiester analogue.

EXAMPLE 4

Oligonucleotide Having 2'-5' Phosphodiester Olig nucleotid Regions Flanking A Central 2'-Deoxy 3'-5' Phosphorothioate Oligonucleotide Region

For the preparation of a 20 mer oligonucleotide, a first region of 6 RNA nucleotides having 2'-5' linkages is prepared as per the method of Kierzek, *et. al.*, *Nucleic Acids Research* **1992**, *20*, 1685 on a DNA synthesizer utilizing the general protocols of this reference. Upon completion of the 2'-5' linked region, a 2'-deoxy phosphorothioate region of 3'-5' linked DNA oligonucleotide 8 nucleotides long is added. A further 6 nucleotide long region of 2'-5' linkages is then added to complete the oligonucleotide having mixed 2'-5' and 3'-5' linkages.

EXAMPLE 5

Macromolecule Having Regions Of Cyclobutyl Surrogate Nucleosides Linked By Phosphodiester Linkages Flanking A Central 2'-Deoxy 3'-5' Phosphorothioate Oligonucleotide Region

For the preparation of a 20 mer oligonucleotide, a first region of 6 cyclobutyl surrogate nucleosides linked by phosphodiester linkages is prepared as per Example 38 of United States patent application 808,201 on a DNA synthesizer utilizing the protocols of this reference. Upon completion of this region, a 2'-deoxy phosphorothioate region of a 3'-5' linked DNA oligonucleotide 8 nucleotides long is added. A further region of 6 cyclobutyl surrogate nucleosides is then added to complete the macromolecule.

EXAMPLE 6

Macromolecule Having Regions Of Carbocyclic Surrogate Nucleosides Linked By Phosphodiester Linkages Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

Carbocyclic nucleosides are prepare as per the review references cited in Borthwick, *et al.*, *Tetrahedron* **1992**, *48*, 571. The resulting carbocyclic nucleosides are blocked with a

dimethoxytrityl blocking group in the normal manner. The corresponding phosphoramidites are prepared in the manner of Example 38 of United States Patent Application 808,201 substituting the carbocyclic nucleosides for the cyclobutyl nucleosides surrogates. For the preparation of a 18 mer oligonucleotide, a first region of 4 carbocyclic nucleosides linked by phosphodiester linkages is prepared on a DNA synthesizer utilizing the protocols of Example 1. Upon completion of this region, a 2′-deoxy phosphorothioate 3′-5′ linked DNA oligonucleotide 8 nucleotides long is added. A further region of 4 carbocyclic nucleotides is added to complete the macromolecule.

EXAMPLE 7

Oligonucleotide Having 4′-Thionucleotide Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

In the manner of Example 6, a region of 4′-thionucleotides is prepared as per the procedures of PCT patent application PCT/US91/02732. Next a region of normal 2′-deoxy phosphorothioate nucleotides are added followed by a further region of the 4′-thionucleotides.

EXAMPLE 8

Macromolecule Having Peptide Nucleic Acids Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first region of peptide nucleic acids is prepared as per PCT patent application PCT/EP/01219. The peptide nucleic acids are prepared from the C terminus towards the N terminus using monomers having protected amine groups. Following completion of the first peptide region, the terminal amine blocking group is removed and the resulting amine reacted with a 3′-C-(formyl)-2′,3′-dideoxy-5′-trityl nucleotide as prepared as per the procedure of Vasseur, *et. al.*, *J. Am. Chem. Soc.* **1992**, *114*, 4006. The condensation of the amine with the aldehyde moiety of the C-formyl nucleoside is effected as per the conditions of th Vasseur, *ibid.*, to yield an intermediate oxime linkage. The oxime linkage is reduced under reductive

alkylation conditions of Vasseur, *ibid.*, with $HCHO/NaBH_3CN/AcOH$ to yield the nucleoside connected to the peptide nucleic acid via an methyl alkylated amine linkage. An internal 2′-deoxy phosphorothioate nucleotide region is then continued from this nucleoside as per the protocols of Example 1. Peptide synthesis for the second peptide region is commenced by reaction of the carboxyl end of the first peptide nucleic acid of this second region with the 5′ hydroxy of the last nucleotide of the DNA region following removal of the dimethoxytrityl blocking group on that nucleotide. Coupling is effected via DEA in pyridine to form an ester linkage between the peptide and the nucleoside. Peptide synthesis is then continued in the manner of patent application PCT/EP/01219 to complete the second peptide nucleic acid region.

EXAMPLE 9

Oligonucleotide Having 2′-Substituted Oligonucleotide Regions Flanking A Central 2′-Deoxy Phosphoroselenate Oligonucleotide Region

An oligonucleotide is prepared as per Example 3 utilizing 2′-O-methyl substituted nucleotides to prepare the flanking regions and oxidization with 3*H*-1,2-benzothiaseleno-3-ol for introducing the seleno moieties in the central region as per the procedure reported by Stawinski, *et al.*, *Tenth International Roundtable: Nucleosides, Nucleotides and Their Biological Evaluation*, September 16-20, 1992, Abstracts of Papers, Abstract 80.

EXAMPLE 10

Oligonucleotide Having 2′-Substituted Oligonucleotide Regions Flanking A Central 2′-Deoxy Phosphorodithioate Oligonucleotide Region

An oligonucleotide is prepared as per Example 3 utilizing 2′-O-aminopropoxy substituted nucleotides to prepare the flanking regions and the procedures of Beaton, *et. al.*, Chapter 5, Synthesis of oligonucleotide phosphorodithioates, page 109, *Oligonucleotides and Analogs, A Practical Approach*,

Eckstein, F., Ed.; The Practical Approach Series, IRL Press, New York, 1991 to prepare the internal phosphorodithioate region.

EXAMPLE 11

Oligonucleotide Having Boranophosphate Linked Oligonucleotide Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

An oligonucleotide is prepared as per Example 3 utilizing the procedures of published patent application PCT/US/06949 to prepare the flanking boranophosphate regions and the procedures of Example 1 to prepare the central 2′-deoxy phosphorothioate region.

EXAMPLE 12

Oligonucleotide Having 2′-Substituted Methyl Phosphonate Linked Oligonucleotide Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

2-Fluoro nucleosides are prepared as per Example 3 and then converted to nucleotides for the preparation of flanking methylphosphonates linkages as per the procedures Miller et. al., Chapter 6, Synthesis of oligo-2′-deoxyribonucleoside methylphosphonates, page 137, *Oligonucleotides and Analogs, A Practical Approach*, Eckstein, F., Ed.; The Practical Approach Series, IRL Press, New York, 1991. The central internal phosphorothioate region is prepared as per Example 1 followed by the addition of a further 2′-O-substituted methylphosphonate region.

EXAMPLE 13

Oligonucleotide Having 2′-Substituted Methyl Phosphotriester Linked Oligonucleotide Regions Flanking Central 2′-Deoxy Phosphodiester Thymidine Oligonucleotide Region

2-Fluoro nucleosides are prepared as per Example 3 and then converted to nucleotides for the preparation of flanking regions of methyl phosphotriester linkages as per the procedures Miller, *et. al.*, *Biochemistry* **1977**, *16*, 1988. A

central internal phosphodiester region having 7 consecutive thymidine nucleotide residues is prepared as per Example 1 followed by the addition of a further 2'-O-substituted methyl phosphotriester region.

EXAMPLE 14

Macromolecule Having Hydroxylamine Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides alternately linked by methylhydroxylamine linkages and phosphodiester linkages is prepared as per the procedure of Vasseur, *ibid.* A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 15

Macromolecule Having Hydrazine Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by methylhydrazine linkages is prepared as per the procedures of the examples of patent application PCT/US92/04294. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 16

Macromolecule Having Methysulfenyl Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by methylsulfenyl linkages is prepared as per the procedures of the examples of patent application PCT/US92/04294. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as

per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 17

Macromolecule Having Ethanediylimino Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by 1,2-ethanediylimino linkages is prepared as per the procedures of the examples of patent application PCT/US92/04294. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 18

Oligonucleotide Having Methylene Phosphonate Linked Oligonucleotide Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by methylene phosphonate linkages is prepared as per the procedure of the examples of patent application PCT/US92/04294. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 19

Macromolecule Having Nitrilomethylidyne Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by nitrilomethylidyne linkages is prepared as per the procedures of the examples of United States patent application 903,160. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further

flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 20

Macromolecule Having Carbonate Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by carbonate linkages is prepared as per the procedure of Mertes, *et al., J. Med. Chem.* **1969**, *12*, 154. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 21

Macromolecule Having Carbamate Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by carbamate linkages is prepared as per the procedure of Gait, *et. al., J. Chem. Soc. Perkin 1* **1974**, 1684. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 22

Macromolecule Having Silyl Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by silyl linkages is prepared as per the procedure of Ogilvie, *et al., Nucleic Acids Res.* **1988**, *16*, 4583. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the

macromolecule.

EXAMPLE 23

Macromolecules Having Sulfide, Sulfoxide and Sulfone Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by sulfide, sulfoxide and sulfone linkages is prepared as per the procedure of Schneider, *et al.*, *Tetrahedron Letters* **1990**, *31*, 335. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 24

Macromolecules Having Sulfonate Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by sulfonate linkages is prepared as per the procedure of Musicki, *et al.*, *J. Org. Chem.* **1991**, *55*, 4231. A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 24

Macromolecules Having Sulfonamide Linked Oligonucleoside Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by sulfonamide linkages is prepared as per the procedure of Kirshenbaum, *et. al.*, *The 5th San Diego Conference: Nucleic Acids: New Frontiers*, Poster abstract 28, November 14-16, **1990.** A central 2'-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to

complete the macromolecule.

EXAMPLE 25

Macromolecules Having Formacetal Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by formacetal linkages is prepared as per the procedure of Matteucci, *Tetrahedron Letters* **1990**, *31*, 2385 or Veeneman, et. al., *Recueil des Trav. Chim.* **1990**, *109*, 449. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 26

Macromolecules Having Thioformacetal Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by thioformacetal linkages is prepared as per the procedure of Matteucci, *et. al., J. Am. Chem. Soc.* **1991**, *113*, 7767 or Matteucci, *Nucleosides & Nucleotides* **1991**, *10*, 231. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 27

Macromolecules Having Morpholine Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by morpholine linkages is prepared as per the procedure of United States Patent Number 5,034,506. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region

having the same linkages as the first region to complete the macromolecule.

EXAMPLE 28

Macromolecules Having Amide Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleosides linked by amide linkages is prepared as per the procedure of United States patent application serial number 703,619 filed May 21, 1991 and related PCT patent application PCT/US92/04305. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 29

Macromolecules Having Ethylene Oxide Linked Oligonucleoside Regions Flanking A Central 2′-Deoxy Phosphodiester Oligonucleotide Region

A first flanking region of nucleosides linked by ethylene oxide linkages is prepared as per the procedure of PCT patent application PCT/US91/05713. A central 2′-O-deoxy phosphodiester oligonucleotide region three nucleotides long is added as per the procedure of Example 1 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 30

Macromolecules Having 3,4-Dihydroxybutyl Linked Nucleobase Regions Flanking A Central 2′-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleobases linked by 3,4-dihydroxybutyl linkages is prepared as per the procedure of Augustyns, *et. al.*, *Nucleic Acids Research* **1991**, *19*, 2587. A central 2′-O-deoxy phosphorothioate oligonucleotide region is added as per the procedure of Example 3 followed by a further

flanking region having the same linkages as the first region to complete the macromolecule.

EXAMPLE 31

Macromolecules Having Dihydroxypropoxymethyl Linked Nucleobase Regions Flanking A Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A first flanking region of nucleobases linked by dihydroxyproproxymethyl linkages is prepared as per the procedure of Schneider, *et al.*, *J. Am. Chem. Soc.* **1990**, *112*, 453. A central 2'-O-deoxy phosphorothioate oligonucleotide region 9 nucleotides long is added as per the procedure of Example 3 followed by a further flanking region having the same linkages as the first region to complete the macromolecule.

PROCEDURE 1

Ras-Luciferase Reporter Gene Assembly

The ras-luciferase reporter genes described in this study were assembled using PCR technology. Oligonucleotide primers were synthesized for use as primers for PCR cloning of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. H-ras gene templates were purchased from the American Type Culture Collection (ATCC numbers 41000 and 41001) in Bethesda, MD. The oligonucleotide PCR primers 5'-ACA-TTA-TGC-TAG-CTT-TTT-GAG-TAA-ACT-TGT-GGG-GCA-GGA-GAC-CCT-GT-3' (sense), SEQ ID NO: 7, and 5'-GAG-ATC-TGA-AGC-TTC-TGG-ATG-GTC-AGC-GC-3' (antisense), SEQ ID NO: 8, were used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers are expected to produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product was gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR

product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene were 5′-GAG-ATC-TGA-AGC-TTG-AAG-ACG-CCA-AAA-ACA-TAA-AG-3′ (sense), SEQ ID NO: 9, and 5′-ACG-CAT-CTG-GCG-CGC-CGA-TAC-CGT-CGA-CCT-CGA-3′ (antisense), SEQ ID NO: 10, were used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers were expected to yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by HindIII and BssHII restriction endonuclease sites. This fragment was gel purified, precipitated, washed and resuspended in water using standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products were digested with the appropriate restriction endonucleases and cloned by three-part ligation into an expression vector containing the steroid-inducible mouse mammary tumor virus promotor MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the insertion of H-ras 5′ sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter.

PROCEDURE 2

Transfection of Cells with Plasmid DNA:

Transfections were performed as described by Greenberg, M.E. in Current Protocols in Molecular Biology, (Ausubel, *et al.*, eds.), John Wiley and Sons, NY, with the following modifications. HeLa cells were plated on 60 mm dishes at 5 x 10^5 cells/dish. A total of 10 μg of DNA was added to each dish, of which 9 μg was ras-luciferase reporter plasmid and 1 μg was a vector expressing the rat glucocorticoid receptor

under control of the constitutiv Rous sarcoma virus (RSV) promoter. Calcium phosphate-DNA coprecipitates were removed after 16-20 hours by washing with Tris-buffered saline [50 Mm Tris-Cl (pH 7.5), 150 mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum was then added to the cells. At this time, cells were pre-treated with antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

PROCEDURE 3

Oligonucleotide Treatment of Cells:

Immediately following plasmid transfection, cells were washed three times with Opti-MEM (Gibco), prewarmed to 37°C. Two ml of Opti-MEM containing 10 μg/ml N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA) (Bethesda Research Labs, Gaithersburg, MD) was added to each dish and oligonucleotides were added directly and incubated for 4 hours at 37°C. Opti-MEM was then removed and replaced with the appropriate cell growth medium containing oligonucleotide. At this time, reporter gene expression was activated by treatment of cells with dexamethasone to a final concentration of 0.2 μM. Cells were harvested 12-16 hours following steroid treatment.

PROCEDURE 4

Luciferase Assays

Luciferase was extracted from cells by lysis with the detergent Triton X-100, as described by Greenberg, M.E., in Current Protocols in Molecular Biology, (Ausubel, *et al.*, eds.), John Wiley and Sons, NY. A Dynatech ML1000 luminometer was used to measure peak luminescence upon addition of luciferin (Sigma) to 625 μM. For each extract, luciferase assays were performed multiple times, using differing amounts of extract to ensure that the data were gathered in the linear rang of the assay.

PROCEDURE 5

Antis nse Oligonucleotide Inhibition of ras-Luciferase Gene Expression

A series of antisense phosphorothioate oligonucleotide analogs targeted to the codon-12 point mutation of activated H-ras were tested using the ras-luciferase reporter gene system described in the foregoing examples. This series comprised a basic sequence and analogs of that basic sequence. The basic sequence was of known activity as reported in patent application serial number 07/715,196 identified above. In both the basic sequence and its analogs, each of the nucleotide subunits incorporated phosphorothioate linkages to provide nuclease resistance. Each of the analogs incorporated nucleotide subunits that contained 2′-O-methyl substitutions and 2′-deoxy-erythro-pentofuranosyl sugars. In the analogs, a sub-sequence of the 2′-deoxy-erythro-pentofuranosyl sugar containing subunits were flanked on both ends by sub-sequences of 2′-O-methyl substituted subunits. The analogs differed from one another with respect to the length of the sub-sequence of the 2′-deoxy-erythro-pentofuranosyl sugar containing nucleotides. The length of these sub-sequences varied by 2 nucleotides between 1 and 9 total nucleotides. The 2′-deoxy-erythro-pentofuranosyl nucleotide sub-sequences were centered at the point mutation of the codon-12 point mutation of the activated ras.

The base sequences, sequence reference numbers and sequence ID numbers of these oligonucleotides (all are phosphorothioate analogs) are shown in Table 1. In this table those nucleotides identified with a "M" contain a 2′-O-methyl substituent group and the remainder of the nucleotides identified with a "d" are 2′-deoxy-erythro-pentofuranosyl nucleotides.

WEST

TABLE 1

Oligo ref. no.	Sequence	SEQ ID NO:
2570	$C_dC_dA_d$ $C_dA_dC_d$ $C_dG_dA_d$ $C_dG_dG_d$ $C_dG_dC_d$ C_dC_d	1
3975	$C^MC^MA^M$ $C^MA^MC^M$ $C^MG^MA_d$ $C^MG^MG^M$ $C^MG^MC^M$ C^MC^M	2
3979	$C^MC^MA^M$ $C^MA^MC^M$ $C^MG_dA_d$ $C_dG^MG^M$ $C^MG^MC^M$ C^MC^M	3
3980	$C^MC^MA^M$ $C^MA^MC^M$ $C_dG_dA_d$ $C_dG_dG^M$ $C^MG^MC^M$ C^MC^M	4
3985	$C^MC^MA^M$ $C^MA^MC_d$ $C_dG_dA_d$ $C_dG_dG_d$ $C^MG^MC^M$ C^MC^M	5
3984	$C^MC^MA^M$ $C^MA_dC_d$ $C_dG_dA_d$ $C_dG_dG_d$ $C_dG^MC^M$ C^MC^M	6

Figure 1 shows dose-response data in which cells were treated with the phosphorothioate oligonucleotides of Table 1. Oligonucleotide 2570 is targeted to the codon-12 point mutation of mutant (activated) H-ras RNA. The other nucleotides have 2′-O-methyl substituents groups thereon to increase binding affinity with sections of various lengths of inter-spaced 2′-deoxy-erythro-pentofuranosyl nucleotides. The control oligonucleotide is a random phosphorothioate oligonucleotide analog, 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. As the figure shows, treatment of cells with increasing concentrations of oligonucleotide 2570 resulted in a dose-dependent inhibition of ras-luciferase activity in cells expressing the mutant form of ras-luciferase. Oligonucleotide 2570 displays an approximate threefold selectivity toward the mutant form of ras-luciferase as compared to the normal form.

As is further seen in Figure 1, each of the oligonucleotides 3980, 3985 and 3984 exhibited greater inhibition of ras-luciferase activity than did oligonucleotide 2570. The greatest inhibition was displayed by oligonucleotide 3985 that has a sub-sequence of 2′-deoxy-erythro-pentofuranosyl nucleotides seven nucleotides long. Oligonucleotide 3980, having a five nucleotide long 2′-deoxy-erythro-pentofuranosyl nucleotide sub-sequence exhibited the next greatest inhibition followed by oligonucleotide 3984 that has a nine nucl otide 2′-deoxy-erythro-pentofuranosyl nucleotid sub-sequence.

Figure 2 shows the results similar to Figure 1 except it is in bar graph form. Further seen on Figure 2 is the activity of oligonucleotide 3975 and oligonucleotide 3979. These oligonucleotides have sub-sequences of 2′-deoxy-erythro-pentofuranosyl nucleotides one and three nucleotides long, respectively. As is evident from Figure 2 neither of the oligonucleotides having either the one nor the three 2′-deoxy-erythro-pentofuranosyl nucleotide sub-sequences showed significant activity. There was measurable activity for the three nucleotide sub-sequence oligonucleotide 3979 at the highest concentration dose.

The increases in activity of oligonucleotides 3980, 3985 and 3984 compared to oligonucleotide 2570 is attributed to the increase in binding affinity imparted to these compounds by the 2′-O-methyl substituent groups located on the compounds and by the RNase H activation imparted to these compounds by incorporation of a sub-sequence of 2′-deoxy-erythro-pentofuranosyl nucleotides within the main sequence of nucleotides. In contrast to the active compounds of the invention, it is interesting to note that sequences identical to those of the active oligonucleotides 2570, 3980, 3985 and 3984 but having phosphodiester linkages in stead of the phosphorothioate linkages of the active oligonucleotides of the invention showed no activity. This is attributed to these phosphodiester compounds being substrates for nucleases that degrade such phosphodiester compounds thus preventing them potentially activating RNase H.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Gapped 2' Modified Oligonucleotides

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: n/a

(B) FILING DATE: herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCACACCGAC GGCGCCC17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: no

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACATTATGCT AGCTTTTTGA GTAAACTTGT GGGGCAGGAG ACCCTGT47

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iv) ANTI-SENSE: no

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAGATCTGAA GCTTCTGGAT GGTCAGCGC29

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iv) ANTI-SENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
GAGATCTGAA GCTTGAAGAC GCCAAAAACA TAAAG35

(2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iv) ANTI-SENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
ACGCATCTGG CGCGCCGATA CCGTCGACCT CGA33

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iv) ANTI-SENSE: yes
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
GCGTTTTTTTTTTGCG16

(2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iv) ANTI-SENSE: yes
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
CGCAAAAAAAAAACGC16

WHAT IS CLAIMED IS:

1. An oligonucleotide comprising a sequence of nucleotide units capable of specifically hybridizing to a strand of nucleic acid, wherein:

at least one of said nucleotide units is functionalized to increase nuclease resistance of said oligonucleotide;

at least one of said nucleotide units bears a substituent group that increases binding affinity of said oligonucleotide to said strand of nucleic acid; and

a plurality of said nucleotide units have 2′-deoxy-erythro-pentofuranosyl sugar moieties, said 2′-deoxy-erythro-pentofuranosyl nucleotide units being consecutively located in said sequence of nucleotide units.

2. The oligonucleotide of claim 1 wherein said substituent group for increasing binding affinity comprises a 2′-substituent group.

3. The oligonucleotide of claim 2 wherein said 2′-substituent group is fluoro, C1-C9 alkoxy, C1-C9 aminoalkoxy, allyloxy, imidazolealkoxy and poly(ethylene glycol).

4. The oligonucleotide of claim 1 wherein each of said nucleotide units is a phosphorothioate or phosphorodithioate nucleotide.

5. The oligonucleotide of claim 1 wherein the 3′ terminal nucleotide unit of said oligonucleotide includes a nuclease resistance modifying group on at least one of the 2′ or the 3′ positions of said nucleotide unit.

6. The oligonucleotide of claim 1 wherein:

a plurality of said nucleotide units bear substituent groups that increases binding affinity of said oligonucleotide to said strand of nucleic acid, said substituent-bearing nucleotides being divided into a first nucleotide unit sub-sequence and a second nucleotide unit sub-sequence; and

said plurality of 2′-deoxy-erythro-pentofuranosyl nucleotide units is positioned in said sequence of nucleotide units between said first nucleotide unit sub-sequence and said second nucleotide unit sub-sequence.

7. The oligonucleotide of claim 1 wherein:

a plurality of said nucleotide units bear substituent groups that increase binding affinity of said oligonucleotide to said complementary strand of nucleic acid; and

at least a portion of said substituent-bearing nucleotide are consecutively located at one of the 3′ terminus or the 5′ terminus of said oligonucleotide.

8. The oligonucleotide of claim 1 wherein at least five of said nucleotide units have 2′-deoxy-erythro-pentofuranosyl sugar moieties, said at least five 2′-deoxy-erythro-pentofuranosyl nucleotide units being consecutively located in said sequence of nucleotide units.

9. The oligonucleotide of claim 1 wherein from one to about eight of said nucleotide units bear a substituent group that increases the binding affinity of said oligonucleotide to said complementary strand, said substituent-bearing nucleotide units being consecutively located in said sequence of nucleotide units.

10. The oligonucleotide of claim 1 wherein:

from one to about eight of said nucleotide units bear a substituent group for increasing the binding affinity of said oligonucleotide to said complementary strand, said substituent-bearing nucleotide units being consecutively located in said sequence of nucleotide units; and

at least five of said nucleotide units have 2′-deoxy-erythro-pentofuranosyl sugar moieties, said at least five 2′-deoxy-erythro-pentofuranosyl nucleotide units being consecutively located in said sequence of nucleotide units.

11. An oligonucleotide comprising a sequence of phosphorothioate nucleotides capable of specifically hybridizing to a strand of nucleic acid, wherein:

a plurality of said nucleotides bear a substituent group that increases binding affinity of said oligonucleotide to said strand of nucleic acid; and

a plurality of said nucleotides have 2′-deoxy-erythro-pentofuranosyl sugar moieties.

12. The oligonucleotide of claim 11 wherein said substituent group for incr asing binding affinity comprises a

2′-substituent group.

13. The oligonucleotide of claim 12 wherein said 2′-substituent group is fluoro, C1-C9 alkoxy, C1-C9 aminoalkoxy or allyloxy.

14. The oligonucleotide of claim 12 including: a further plurality of said nucleotides bearing 2′-substituent groups;

said 2′-deoxy-erythro-pentofuranosyl nucleotides being positioned in said oligonucleotide between groups of nucleotides having said 2′-substituent group located thereon.

15. The oligonucleotide of claim 11 wherein said substituent-bearing nucleotides are located at one of the 3′ terminus or the 5′ terminus of said oligonucleotide.

16. An oligonucleotide comprising a sequence of phosphorothioate nucleotides capable of specifically hybridizing to a strand of nucleic acid, wherein:

a first portion of said nucleotides have 2′-deoxy-2′-fluoro, 2′-methoxy, 2′-ethoxy, 2′-propoxy, 2′-aminopropoxy or 2′-allyloxy pentofuranosyl sugar moieties; and

a further portion of said nucleotides have 2′-deoxy-erythro-pentofuranosyl sugar moieties.

17. An oligonucleotide of claim 16 wherein said first portion of said nucleotides are located at either the 3′ terminus or the 5′ terminus of said oligonucleotide.

18. An oligonucleotide of claim 17 including:

an additional portion of said nucleotides having 2′-deoxy-2′-fluoro, 2′-methoxy, 2′-ethoxy, 2′-propoxy, 2′-aminopropoxy or 2′-allyloxy pentofuranosyl sugar moieties; and

said further portion of said nucleotides positioned in said oligonucleotide between said first portion of nucleotides and said additional portion of said nucleotides.

19. A method of treating an organism having a disease characterized by the undesired production of a protein comprising contacting the organism with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a strand of nucl ic acid coding for said protein at least one of the nucleotides being functionalized to

increase nuclease resistance of the oligonucleotide, a plurality of the nucleotides having a substituent group located thereon to increase binding affinity of the oligonucleotide to the strand of nucleic acid, and a plurality of the nucleotides having 2′-deoxy-erythro-pentofuranosyl sugar moieties.

20. The method of claim 19 wherein each of said nucleotides is a phosphorothioate nucleotide.

21. The method of claim 19 wherein said substituent group is a 2′-substituent group.

22. The method of claim 21 wherein said 2′-substituent group is fluoro, alkoxy, aminoalkoxy or allyloxy.

23. A pharmaceutical composition comprising:

an pharmaceutically effective amount of an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a strand of nucleic acid, at least one of the nucleotides being functionalized to increase nuclease resistance of the oligonucleotide, a plurality of the nucleotides having a substituent group located thereon to increase binding affinity of the oligonucleotide to a complementary strand of nucleic acid; a plurality of the nucleotides having 2′-deoxy-erythro-pentofuranosyl sugar moieties; and

a pharmaceutically acceptable diluent or carrier.

24. A method of modifying *in vitro* a sequence-specific nucleic acid, comprising contacting a test solution containing RNase H and said nucleic acid with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a strand of nucleic acid where at least one of the nucleotides is functionalized to increase nuclease resistance of the oligonucleotide, where a plurality of the nucleotides have a substituent group located thereon to increase binding affinity of the oligonucleotide to a complementary strand of nucleic acid, and where a plurality of the nucleotides have 2′-deoxy-erythro-pentofuranosyl sugar moieties.

25. A method of concurrently enhancing hybridization and RNase H activation in a organism comprising contacting the

organism with an oligonucleotide having a sequence of nucleotides capable of specifically hybridizing to a complementary strand of nucleic acid and where at least one of the nucleotides is functionalized to increase nuclease resistance of the oligonucleotide, where a plurality of the nucleotides have a substituent group located thereon to increase binding affinity of the oligonucleotide to a complementary strand of nucleic acid, and where a plurality of the nucleotides have 2'-deoxy-erythro-pentofuranosyl sugar moieties.

26. A macromolecule comprising a plurality of nucleosides linked by covalent linkages in a sequence that is hybridizable to a complementary nucleic acid, wherein:

said nucleosides are selected from α-nucleosides, β-nucleosides including 2'-deoxy-erythro-pentofuranosyl β-nucleosides, 4'-thionucleosides and carbocyclic-nucleosides;

said linkages are selected from charged phosphorous linkages, neutral phosphorous linkages or non-phosphorous linkages; and

said sequence of linked nucleosides contains at least two nucleoside regions, wherein:

a first of said regions includes nucleosides selected from said α-nucleosides linked by charged and neutral 3'-5' phosphorous linkages, said α-nucleosides linked by charged and neutral 2'-5' phosphorous linkages, said α-nucleosides linked by non-phosphorous linkages, said 4'-thionucleosides linked by charged and neutral 3'-5' phosphorous linkages, said 4'-thionucleosides linked by charged and neutral 2'-5' phosphorous linkages, said 4'-thionucleosides linked by non-phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 3'-5' phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 2'-5' phosphorous linkages, said carbocyclic-nucleosides linked by non-phosphorous linkages, said β-nucleosides linked by charged and neutral 2'-5' linkages, and said β-nucleosides linked by non-

phosphorous linkages; and

a second of said regions consists of said 2'-deoxy-<u>erythro</u>-pentofuranosyl ß-nucleosides linked by charged 3'-5' phosphorous linkages having a negative charge at physiological pH.

27. A macromolecule of claim 26 wherein said second region includes at least 3 of said 2'-deoxy-<u>erythro</u>-pentofuranosyl ß-nucleosides.

28. A macromolecule of claim 26 wherein said second nucleoside region is position between said first nucleoside region and a third nucleoside region, said third nucleoside region including nucleosides selected from said α-nucleosides linked by charged and neutral 3'-5' phosphorous linkages, said α-nucleosides linked by charged and neutral 2'-5' phosphorous linkages, said α-nucleosides linked by non-phosphorous linkages, said 4'-thionucleosides linked by charged and neutral 3'-5' phosphorous linkages, said 4'-thionucleosides linked by charged and neutral 2'-5' phosphorous linkages, said 4'-thionucleosides linked by non-phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 3'-5' phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 2'-5' phosphorous linkages, said carbocyclic-nucleosides linked by non-phosphorous linkages, said ß-nucleosides linked by charged and neutral 2'-5' linkages, and said ß-nucleosides linked by non-phosphorous linkages.

29. A macromolecule of claim 26 wherein said charged phosphorous linkages include phosphodiester, phosphorothioate, phosphorodithioate, phosphoroselenate or phosphorodiselenate linkages.

30. A macromolecule of claim 26 wherein said charged phosphorous linkages is phosphodiester or phosphorothioate.

31. A macromolecule of claim 26 wherein said neutral phosphorous linkages include alkyl and aryl phosphonates, alkyl and aryl phosphoroamidites, alkyl and aryl phosphotriesters, hydrogen phosphonate and boranophosphate linkages.

32. A macromolecule of claim 26 wherein said non-

phosphorous linkages include peptide linkages, hydrazine linkages, hydroxy-amine linkages, carbamate linkages, morpholine linkages, carbonate linkages, amide linkages, oxymethyleneimine linkages, hydrazide linkages, silyl linkages, sulfide linkages, disulfide linkages, sulfone linkages, sulfoxide linkages, sulfonate linkages, sulfonamide linkages, formacetal linkages, thioformacetal linkages, oxime linkages and ethylene glycol linkages.

33. A macromolecule of claim 26 wherein said first nucleoside region includes at least two α-nucleoside linked by a charged or neutral 3′-5′ phosphorous linkages.

34. A macromolecule comprising a plurality of units linked by covalent linkages in a sequence that is hybridizable to a complementary nucleic acid, wherein:

said units are selected from nucleosides and nucleobases:

said nucleosides are selected from α-nucleosides, β-nucleosides including 2′-deoxy-erythro-pentofuranosyl β-nucleosides, 4′-thionucleosides, and carbocyclic-nucleosides;

said nucleobases are selected from purin-9-yl and pyrimidin-1-yl heterocyclic bases;

said linkages are selected from charged 3′-5′ phosphorous, neutral 3′-5′ phosphorous, charged 2′-5′ phosphorous, neutral 2′-5′ phosphorous or non-phosphorous linkages; and

said sequence of linked units is divided into at least two regions, wherein:

a first of said regions includes said nucleobases linked by non-phosphorous linkages and nucleobases that are attached to phosphate linkages via non-sugar tethering groups, and nucleosides selected from said α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said α-nucleosides linked by non-phosphorous linkages, said 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said 4′-thionucleosides

linked by charged and neutral 2′-5′ phosphorous linkages, said 4′-thionucleosides linked by non-phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said carbocyclic-nucleosides linked by non-phosphorous linkages, said β-nucleosides linked by charged and neutral 2′-5′ linkages, and said β-nucleosides linked by non-phosphorous linkages; and

a second of said regions includes said 2′-deoxy-<u>erythro</u>-pentofuranosyl β-nucleosides linked by charged 3′-5′ phosphorous linkages having a negative charge at physiological pH.

35. The macromolecule of claim 34 wherein said first region includes at least two nucleobases linked by a non-phosphate linkage.

36. The macromolecule of claim 35 wherein said non-phosphate linkage is a peptide linkage.

37. The macromolecule of claim 35 wherein said second region is positioned between said first region and a third region, said third region including said nucleobases linked by non-phosphorous linkages and nucleobases that are attached to phosphate linkages via a non-sugar tethering moiety, and nucleosides selected from said α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said α-nucleosides linked by non-phosphorous linkages, said 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said 4′-thionucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said 4′-thionucleosides linked by non-phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said carbocyclic-nucleosides linked by non-phosphorous linkages, said β-nucleosides linked by charged and neutral 2′-5′ linkages, and said β-nucleosides linked by

non-phosphorous linkages.

38. A macromolecule of claim 35 wherein said nucleobases are selected from adenine, guanine, cytosine, uracil, thymine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl adenine, 2-propyl and other alkyl adenine, 5-halo uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo, amino, thiol, thiolalkyl, hydroxyl and other 8 substituted adenine and guanine, or 5-trifluoromethyl uracil and cytosine.

39. A macromolecule comprising a plurality of units linked by covalent linkages in a sequence that is hybridizable to a complementary nucleic acid, wherein:

said units are selected from nucleosides and nucleobases;

said nucleosides are selected from α-nucleosides, ß-nucleosides, 4′-thionucleosides and carbocyclic-nucleosides;

said nucleobases are selected from purin-9-yl and pyrimidin-1-yl heterocyclic bases;

said linkages are selected from charged phosphorous, neutral phosphorous or non-phosphorous linkages; and

said sequence of linked units is divided into at least two regions, wherein:

a first of said regions includes said α-nucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said α-nucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said α-nucleosides linked by non-phosphorous linkages, said 4′-thionucleosides linked by charged and neutral 3′-5′ phosphorous linkages, said 4′-thionucleosides linked by charged and neutral 2′-5′ phosphorous linkages, said 4′-thionucleosides linked by non-phosphorous linkages, said carbocyclic-nucleosides linked by charged and neutral phosphorous linkages, said carbocyclic-nucleosides linked by non-phosphorous linkages, said ß-nucleosides linked by charged and neutral 3′-5′ linkages, said ß-nucleosides linked by charged and neutral 2′-5′

linkages, and said ß-nucleosides linked by non-phosphorous linkages; and

a second of said regions including said nucleobases linked by non-phosphorous linkages and nucleobases that are attached to phosphate linkages via a non-sugar tethering moiety.

40. The macromolecule of claim 38 wherein said non-phosphate linkage is a peptide linkage.

41. A macromolecule of claim 38 including a plurality of said first regions.

42. A macromolecule of claim 38 including a plurality of said second regions.

43. A macromolecule of claim 41 including a plurality of said first regions.

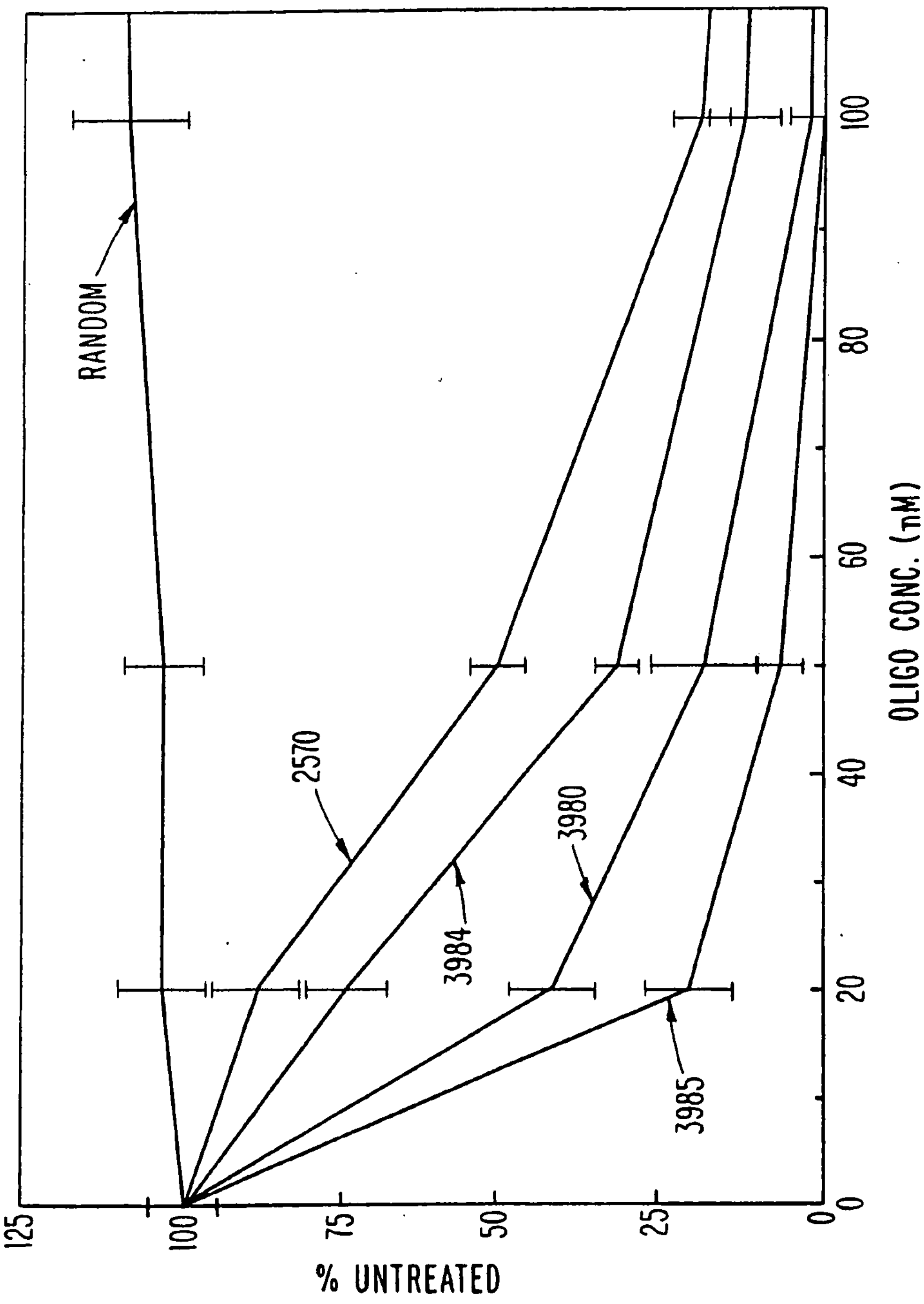
44. A method of treating an organism having a disease characterized by the undesired production of a protein comprising contacting the organism with a compound of claim 34.

45. A pharmaceutical composition comprising a pharmaceutically effective amount of a compound of claim 34 and a pharmaceutically acceptable diluent or carrier.

46. A method of modifying *in vitro* a sequence-specific nucleic acid, comprising contacting a test solution containing a RNase H and said nucleic acid with a compound of claim 34.

47. A method of treating an organism having a disease characterized by the undesired production of a protein comprising contacting the organism with a compound of claim 39.

48. A pharmaceutical composition comprising a pharmaceutically effective amount of a compound of claim 39 and a pharmaceutically acceptable diluent or carrier.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3984
3980
3985
125
100
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50
25
0
% UNTREATED
0
20
40
60
80
100
OLIGO CONC. (nM)

Fig. 1

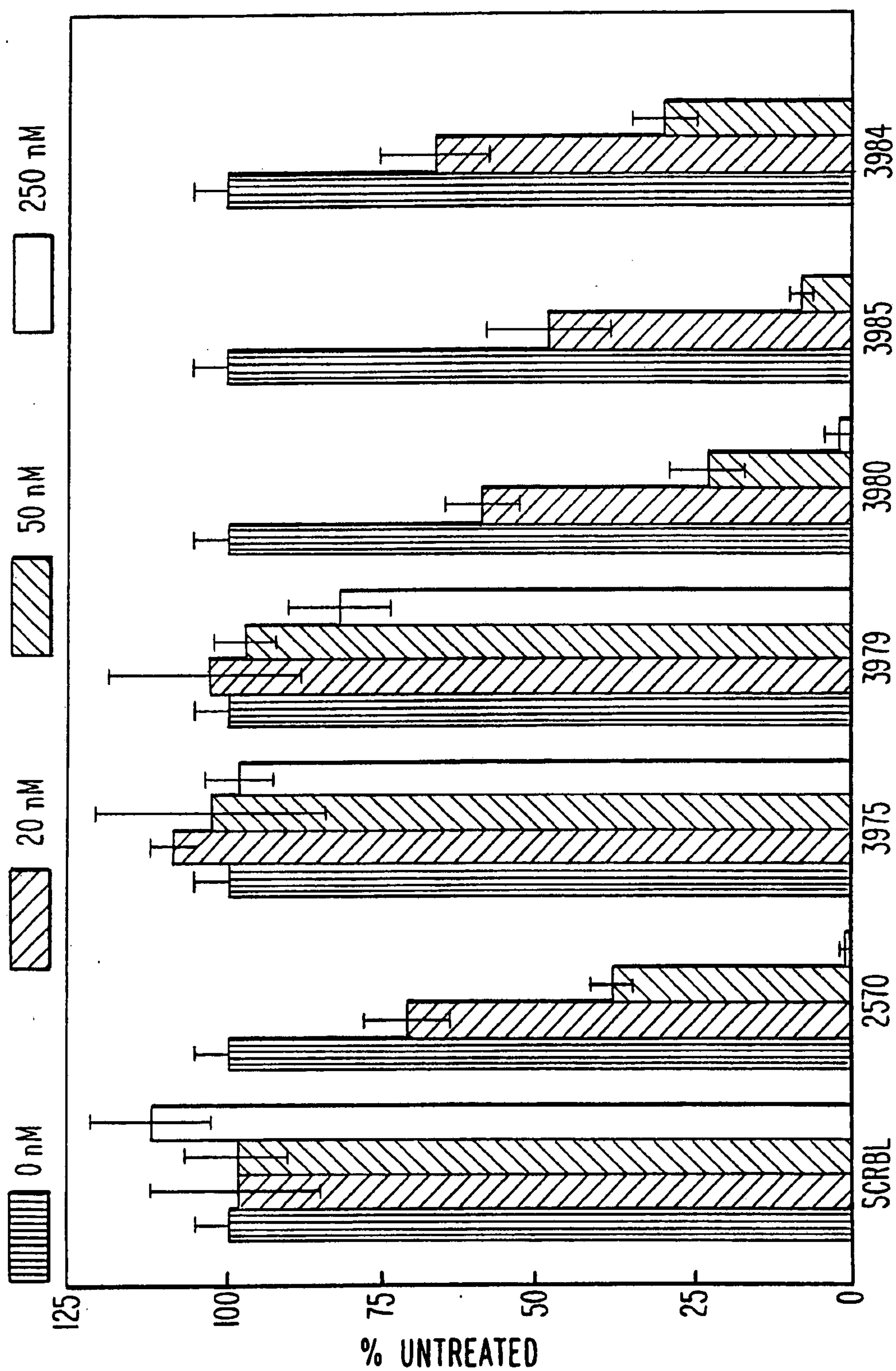
0 nM
20 nM
50 nM
250 nM
% UNTREATED
0
25
50
75
100
125
SCRBL
2570
3975
3979
3980
3985
3984

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/11339

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 21/04
US CL :536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.2, 23.4, 23.5; 435/6, 91; 514/44,; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, CLAIMS FILE, WORLD PATENT INDEX, CHEMICAL ABSTRACT ON-LINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Annual Reports in Medicinal Chemistry, 23, Chapter 30, copyright 1988, MILLER ET AL., "Oligonucleotide Inhibitors of Gene Expression in Living Cells: New Opportunities in Drug Design", pages 295-304. See the entire document.	1-48
Y	Proceedings of the National Academy of Science, USA, Vol. 85, issued October 1988, AGRAWAL ET AL., "Oligodeoxynucleoside Phosphoramidates and Phosphorothioates as inhibitors of Human Immunodeficiency Virus", pages 7079-7083. See the abstract.	1-23
Y	The EMBO Journal, Vol. 10, No. 5, issued 1991, Saison-Behmoaras et al., "Short modified anti-sense oligonucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation," pages 1111-1118. See the entire document.	24-25
Y	Nucleic Acids Research, Vol. 19, No. 8, issued 1991, DAGLE ET	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
13 APRIL 1993	30 APR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer AMELIA B. YARBROUGH Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Tetrahedron Letters, Vol. 31, No. 6, issued 1990, PETERSEN ET AL., "Chemical Synthesis of Dimer Ribonucleotides Containing Internucleotidic Phosphoradithioate Linkages", pages 911-914. See pages 911-912.	1-48
Y	Antisense Research and Development, No. 1, issued 1991, DAGLE ET AL., "Pathways of Degradation and Mechanism of Action of Antisense Oligonucleotides in Xenopus laevis Embryos", pages 11-20. See the abstract.	1-48
Y	Anti-Cancer Drug Design, No. 2, issued 1987, MILLER ET AL., "A new approach to chemotherapy based on molecular biology and nucleic acid chemistry: Matagen (masking tape for gene expression)", pages 117-128. See the summary.	1-48
Y	Cohen, "OLIGODEOXYNUCLEOTIDES", published 1989, by CRC Press, INC, Boca Raton, (F1), pages 1-255, Note pages 16, 35, 36, 38, 55, 62, 66, 67, 79-82, 85.	1-48
Y	The Journal of the American Chemical Society, Vol. 113, issued 1991, BRILL ET AL., "Synthesis of Deoxydinucleotide Phosphorodithioates", pages 3972-3980. See pages 3972-3973.	1-48
O,Y	KAWASAKI ET AL., disclosed January 1991, "Synthesis and Biophysical Studies of 2'-dR1B0-2'-F Modified Oligonucleotides", pages 1-9. See entire document.	1-48
Y	Nucleic Acid Research, Vol. 18, No. 16, issued 1990, DAGLE ET AL., "Targeted degradation of mRNA in Xenopus oocytes and embryos directed by modified oligonucleotides: Studies of An2 and Cyclin in Embryogenesis", page 4751-4757. See 4751-4752.	1-48
Y	WO, A, 91/12323 (PEDERSON ET AL.) 22 August 1991, see the entire document.	1-48

INTERNATIONAL SEARCH REPORT	International application No. PCT/US92/11339

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they [illegible] to parts of the i[illegible]ional application that do not comply with the prescribed requirements to such an extent th[illegible]aningful inter[illegible] se[illegible]ch can be carried out, specifically:

3. ☐ Claims Nos..
because they [illegible] ent claims [illegible] afted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations [illegible]ity of inve[illegible]ng (Continuation of item 2 of first sheet)

This International Sea[illegible]uthority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fe[illegible] were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be s[illegible]arched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
(telephone practice) 1-23

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/11339

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-23, drawn to an oligonucleotide which contains at least one nucleotide that increases nuclease resistance and at least one that increases binding affinity, method of treating using said oligonucleotide and a pharmaceutical composition containing said compound, classified in class 536, subclass 23.1.

Group II, claims 24-25, drawn to a method of modifying RNA, classified in class 435, subclass 6.

Group III, claims 26-48, drawn to macromolecule, methods of treating with them and pharmaceutical composition containing them, classified in class 536, subclass 23.5